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Purification and Biochemical Characterization of the Nuclear Sperm-Specific Proteins of the Bivalve Mollusks *Agriodesma saxicola*[1] and *Mytilimeria nuttalli*

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Abstract. The proteins from the nuclei of the sperm from two different species of the subclass Anomalodesmata of the class Bivalvia have been analyzed for the first time. In both instances—*Agriodesma saxicola* (Baird, 1863) and *Mytilimeria nuttalli* (Conrad, 1837)—the compositional pattern is very similar. The sperm chromatin is organized by a major protamine-like PL-I protein. As in all PL-I, this protein has a trypsin-resistant core. In both species analyzed, PL-I contains cysteine residues that account for the presence of the monomer (M) and dimer (D) forms observed in the total nuclear HCl extracts. The molecular mass of these proteins is 21,000 Da in *A. saxicola,* and 25,000 Da in *M. nuttalli.* All of the specimens of *A. saxicola* analyzed were hermaphrodites. As a result, the nuclear sperm-specific proteins from several preparations were readily and extensively degraded by protease activity from the oocytes. Such degradation was always observed when cross contamination between the two gonadal tissues accidentally occurred during protein extraction.

Introduction

The presence of a highly specialized histone H1 (PL-I protein) seems to be a common feature of the nuclear protein composition of the sperm of bivalve mollusks (Jutglar *et al.*, 1991). Despite the structural heterogeneity of the sperm proteins within this taxonomic group (Ausió, 1986; Zalensky and Zalenskaya, 1980), a PL-I protein has been identified in each of the species analyzed in detail so far. Like histone H1, this protein is soluble in diluted perchloric acid, has a globular trypsin-resistant core, is lysine rich, and yet is compositionally related to the protamines (PL = protamine-like) (Subirana *et al.*, 1973). Of all the different nuclear sperm-specific proteins found within a given species, PL-I is the one with the lowest electrophoretic mobility in urea-acetic acid gels (Ausió, 1986). The presence of a protamine-like histone H1-like protein in bivalve mollusks may have important evolutionary implications, not only within the phylum Mollusca (Subirana and Colom, 1987), but also within other taxonomic groups.

As pointed out by Kasinsky (1989), however, only some of the subclasses within the class Bivalvia have been analyzed so far, and only a few species have been thoroughly characterized. Nevertheless, at least one sperm-specific histone H1 (PL-I) protein has been identified: in *Mytilus californianus* (by Jutglar *et al.*, 1991), *Crassostrea gigas* (by Sellos, 1985), and *Glycymeris yesonensis* (by Odintsova *et al.*, 1989) (subclass Pteriomorphia); in *Ensis minor* (by Giancotti *et al.*, 1983), *Spisula solidissima* (by Ausió *et al.*, 1987) and *Macoma nasuta* (Ausió, 1988) (Subclass Heterodonta); and in *Anodonta piscinallis* (by Rozov *et al.*, 1984) (Subclass Palaeoheterodonta). In some of these species, two histone H1-like proteins have been described.

Although the subclass Heterodonta has been widely studied (Ausió, 1986), three subclasses, according to Barnes's (1980) classification of the bivalve mollusks, have never been characterized: Cryptodonta, Palaeotaxodonta, and Anomalodesmata. In the present work, we have analyzed and characterized the sperm-specific proteins of two species within the subclass Anomalodesmata and have shown that each contains a highly specialized histone H1-

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[1] This species is more commonly referred to as *Entodesma saxicola,* but see Bernard, 1983.

like (PL-I) protein that is the major protein component of the nuclei of the sperm of that organism.

Materials and Methods

Living specimens

Specimens of *Agriodesma saxicola* and *Mytilimeria nuttalli* were collected along the west coast of Vancouver Island, British Columbia, Canada, by SCUBA divers from the Biology Department at the University of Victoria.

Nuclei preparation and protein extraction

Isolation of sperm nuclei and HCl crude extraction of the nuclear basic proteins was performed as described elsewhere (Ausió, 1986). Briefly, after carefully opening the shell, a small incision was made in the gonadal tissue, and the spontaneously released sperm were resuspended in NaCl 0.15 *M*, Tris-HCl 20 m*M* pH 7.6, 0.2 m*M* PMSF (Phenylmethylsulphonyl Fluoride) (buffer A). Because *A. saxicola* is hermaphroditic, its sperm would sometimes be contaminated with oocytes released accidentally from the intimately associated ovaries (see below for discussion).

The sperm suspension was centrifuged at 3000 × *g* for 10 min in a SS-34 Sorvall rotor at 4°C. The pellet obtained was homogenized in buffer A containing 0.5% TritonX-100. After standing for 10 min on ice, the suspension was spun down under the same conditions as before. This step is meant to solubilize most of the cytoplasmic membranes, including the sperm flagella and the acrosome. Notice that this step will also expose the sperm nuclei to egg lysates in those samples of *A. saxicola* contaminated with oocytes; such cytoplasmic contamination may be responsible for the protein degradation observed under these circumstances. The detergent-treated pellet was immediately homogenized in 0.4 *N* HCl. Solubilization was continued for 2 h under stirring at 4°C. Finally, the suspension was centrifuged at 12,000 × *g* for 10 min at 4°C, and the acid extract was precipitated with 6 volumes of acetone, overnight, at −20°C.

Gel electrophoresis

Polyacrylamide gel electrophoresis was carried out on urea-acetic acid gels, as described elsewhere (Ausió, 1986).

Protein purification and fractionation

Ionic exchange chromatography was carried out on a 10 × 100 mm Protein-Pak SP 8HR column from Waters-Millipore as described elsewhere (Mogensen *et al.*, 1991).

Gel filtration was carried out on a 10 × 300 mm FPLC Superose 12HR 10/30 column from Pharmacia. The elution buffer was 6 *M* guanidinium chloride (Gdn-HCl; Schwarz/Mann Biotech), 50 m*M* Tris-HCl pH 7.6.

Reverse phase high pressure liquid chromatography (HPCL)

HPLC was carried out on a 5 μ (25 × 0.46 cm) Vydac C_4 column, with 0.1% trifluoroacetic acid (TFA) as eluant with different acetonitrile gradients.

Determination of the molecular mass

The molecular mass of each protein was determined by gel filtration under denaturing conditions on a Superose 12HR column in the presence of 6 *M* Gdn-HCl (see above). Several protamines, protamine-like proteins, and histones of known molecular mass were used as standards: PL-I from *Spisula solidissima* (Mr: 33,500 Da) (Ausió and Subirana, 1982a); Histone H1 from calf thymus (Mr: 22,000 Da) (DeLange, 1976); PL-III (ϕ1) from *Mytilus edulis* (Mr: 9600 Da) (Ausió and Subirana, 1982b); and unfractionated salmine from *Oncorhynchus* sp. (Mr; 4300 Da) (Ando *et al.*, 1973). Histone H1 from calf thymus was purchased from Worthington, and salmine (sulfate form) was obtained from Sigma. The rest of the protamines were prepared in my laboratory. Globular proteins were also used as a molecular mass markers: bovine serum albumin (Mr: 68,000); ovoalbumin (Mr: 46,000); chymotrypsinogen A (Mr: 25,000); and ribonuclease A (Mr: 13,200). These proteins were purchased from Pharmacia; all of them were subjected to performic acid oxidation before being applied to the column (see below). Vitamin B12 (Mr: 1350) was purchased from Sigma. For the estimation of the molecular mass, the column was calibrated with the above standard proteins, and a plot of K_{av} *versus* log Mr was constructed (Mr = molecular mass; K_{av} = distribution coefficient).

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_o and V_t are the void and total volume of the column, and V_e = the elution volume of a given protein. Blue Dextran and dansyl-L-alanine (Sigma) were used to determine V_o and V_t experimentally. The proteins of unknown Mr were mixed with the protein standards and run together through the column. Their molecular masses were estimated by interpolation of their K_{av} values on the best fitting line of the calibration plot.

Amino acid analysis

Amino acid analysis was carried out on an Applied Biosystems model 420A derivatizer-analyzer system. The hydrolysis was carried out in gas-phase 6 *N* HCl and 1% phenol under an argon atmosphere at 165°C, for 1 h, 2

h, and 4 h, the final amino acid composition was obtained by extrapolation of the data to zero time. So that cysteine could be quantified, all protein samples were pyridylethylated before hydrolysis, as described below.

Chemical modification of proteins

Reduction of SH groups. The SH groups of cysteine were reduced as described by Kuehl (1979). Briefly, the proteins, at 1 mg/ml in 6 *M* urea 20 m*M* Tris-HCl pH 7.6, were reduced in the presence of 8% β-mercaptoethanol for 3 h at room temperature.

Oxidation of SH groups. Oxidation was carried out under the same buffer conditions as above, but in the presence of 0.72 m*M* O-phenanthroline and 0.36 m*M* $CuSO_4$.

Performic acid oxydation. Performic acid was prepared according to Hirs (1967). For the oxidation, 1-mg aliquots of protein were dissolved in 0.5 ml of performic acid, which had been previously cooled on ice. The reaction was allowed to proceed for 4 h in an ice bath in capped tubes. The sample was then resuspended in a 25-fold excess of HPLC grade distilled water and lyophilized.

Cysteine pyridylethylation. Proteins were pyridylethylated, providing for a quantitative estimate of cysteine in the amino acid analysis. The procedure used was as follows: proteins (≅1 nanomol) were dissolved in 44 μl of 6.8 *M* urea, 60 m*M* Tris-HCl, 1.25 m*M* EDTA (pH 7.6), and 2.3% β-mercaptoethanol. The solution was incubated for 3 h at room temperature in the dark. Subsequently, 8

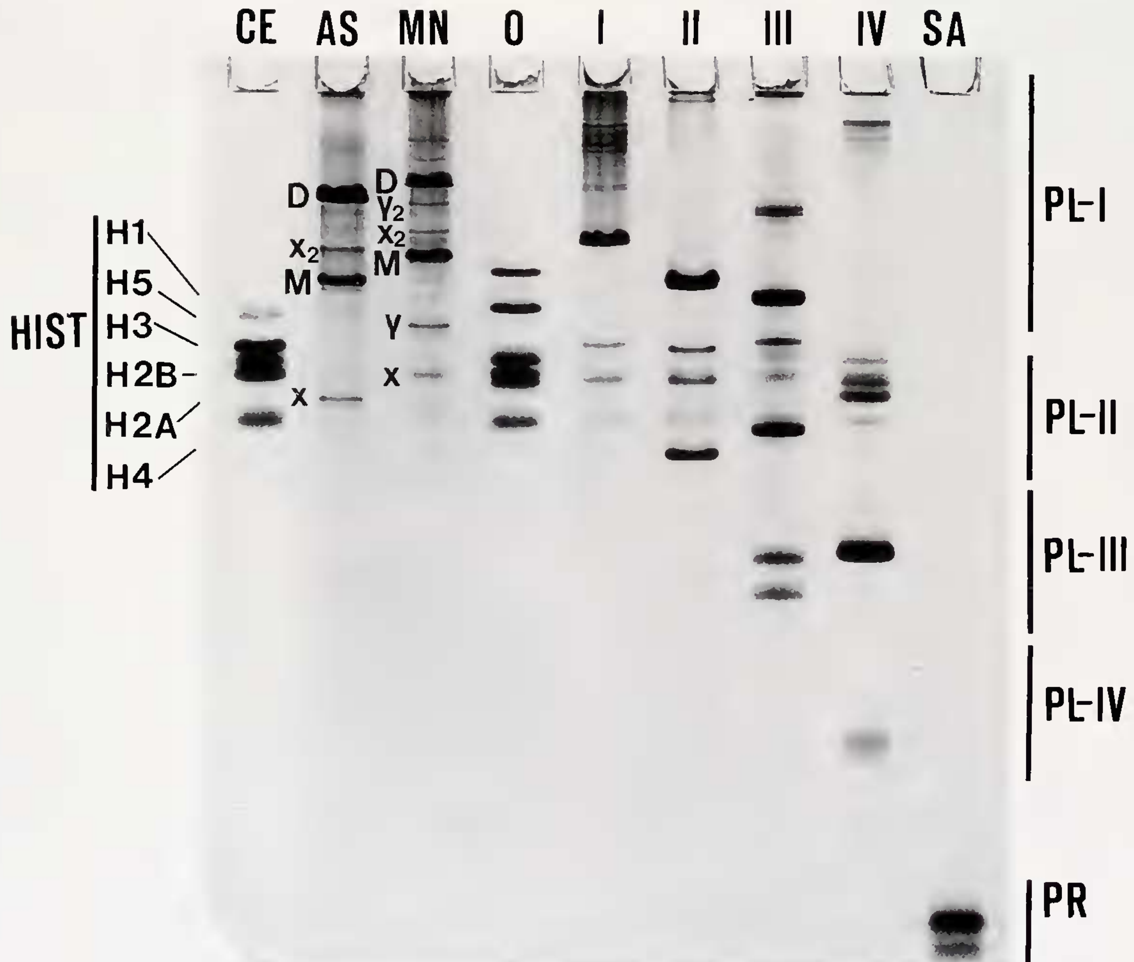

Figure 1. Urea acetic acid PAGE analysis of the nuclear sperm-specific proteins of *Agriodesma saxicola* (AS) and *Mytilimeria nuttalli* (MN) in comparison to a histone standard from chicken erythrocytes (CE) and to a protamine from salmon, salmine (SA). The nuclear sperm-specific proteins of one representative of each of the five groups (O, I, II, III, and IV) of the classification of the bivalve mollusks (Ausió, 1986) are also shown. The representative species chosen for each group were: O: *Pecten maximus;* I: *Spisula solidissima;* II: *Ensis ensis;* III: *Macoma nasuta,* and IV: *Mytilus edulis.* The regions corresponding to the different protamine-like (PL-I, PL-II, PL-III, and PL-IV) proteins defined in Ausió (1986) are also shown. HIST: histone region. PR: protamine. D: dimer form, M: monomer form of the major sperm protein component in each species. X_2, Y_2: possible dimer forms of the minor sperm protein components X, Y.

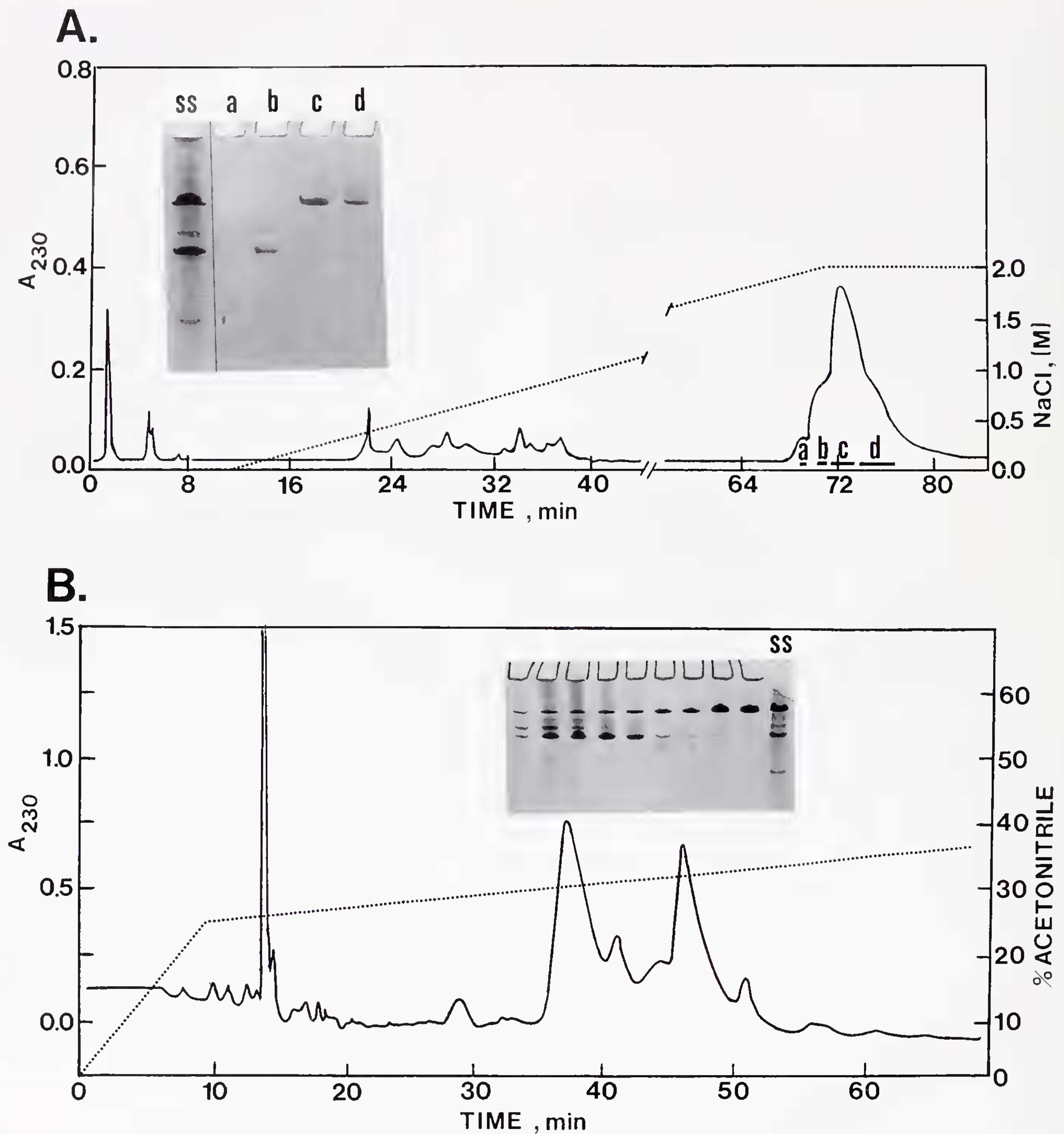

Figure 2. Fractionation of a crude 0.4 *N* HCl extract from the nuclei of the sperm of *Agriodesma saxicola*. (A) Ionic exchange chromatography on a (10 × 100 mm) Protein-Pak SP 8HR column. Proteins were eluted with a linear (0–2 *M*) NaCl gradient in 50 m*M* Na-phosphate buffer (pH 6.8) at a flow rate of 1 ml/min. The inset shows the urea-acetic acid PAGE analysis of the fractions indicated. (B) Reverse-phase HPLC on a Vydac C_4 column. Elution was carried with an acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The inset shows the electrophoretic analysis of the fractionation. The lanes shown in the inset, and the chromatogram has been aligned to match the fraction analyzed with its corresponding position in the chromatogram. SS: starting sample.

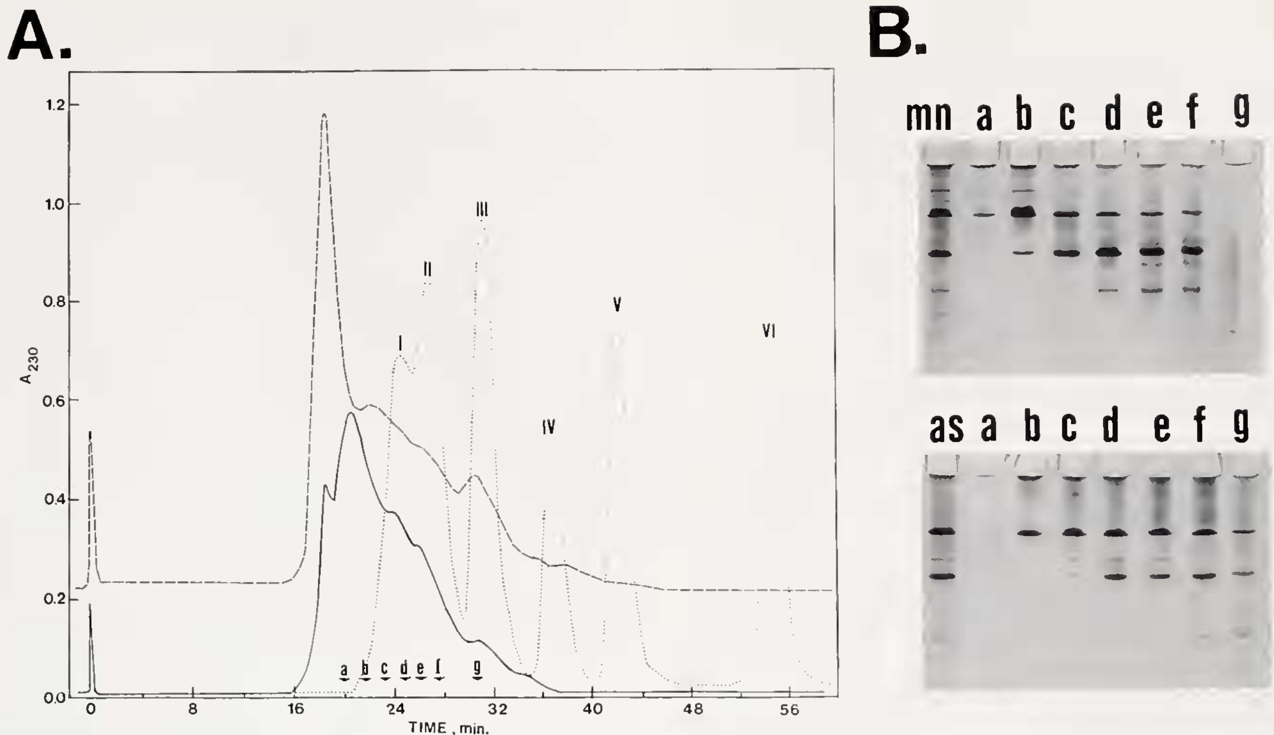

Figure 3. (A) Gel filtration FPLC on a Superose 12 HR 10/30 column. The elution buffer was 6 *M* Gdn-HCl in 50 m*M* Tris-HCl pH 7.6. The flow rate was 0.4 ml/min. The elution profiles of HCl nuclear extracts from the sperm of *Mytilimeria nuttalli* (– – –) and *Agriodesma saxicola* (——) are shown together with the elution profile (· · · ·) of some of the standards used to calibrate this column: I: PL-I from *Spisula solidissima;* II: Histone H1 from calf thymus; III: PL-III from *Mytilus edulis;* IV: protamine salmine; V: vitamin B 12; VI: dansyl-L-alanine. (B) Electrophoretic analysis on urea-acetic acid gels of the fractions a, b, c, d, e, f, g of the elution profiles of *M. nuttalli* and *A. saxicola*. mn: starting sample of *M. nuttalli*, as: starting sample of *A. saxicola*.

μl of 4-vinylpyridine was added, and the reaction was allowed to proceed for 2 h at room temperature. The sample was then immediately desalted in an HPLC reverse phase C_8Vydac column, which was eluted for 5 min with 0.1% TFA (trifluoroacetic acid), and for 20 min with a 0–70% acetonitrile gradient in 0.1% TFA. β-lactoglobulin from Applied Biosystems Inc. was used as a standard for this procedure.

Trypsin digestion. Trypsin digestion of proteins in high salt—2 *M* NaCl, 50 m*M* Na-phosphate buffer (pH 6.8)—was carried out as described elsewhere (Ausió *et al.*, 1987).

Results

Chromatographic analysis and purification of the sperm-specific nuclear proteins from A. saxicola *and* M. nuttalli

Figure 1 shows the 0.4 *N* HCl protein extracts from the nuclei of the sperm of *A. saxicola* and *M. nuttalli*. They are shown in comparison to the five groups previously established for the classification of the nuclear sperm-specific proteins of the bivalve mollusks (Ausió, 1986). In each of the two species analyzed, two major protein bands run in the region of the PL-I proteins (Ausió, 1986). In addition to these proteins, 10–20% of minor protein fractions X and Y, which run in the histone region, are also observed (see Fig. 1-AS and 1-MN). This protein composition was sufficiently novel that the two organisms could not, at first, be assigned to any of the protein groups previously established in my classification of the bivalves (Ausió, 1986). That was not surprising because they belong to a subclass (Anomalodesmata) that had not been analyzed before. I therefore decided to purify and characterize each of the major protein components of these organisms.

The first attempt at fractionation by ionic exchange FPLC under non-denaturing conditions is shown in Figure 2A. Most of the protein components coeluted in a single multiphasic peak at around 2 *M* NaCl, but some protein separation was clearly achieved as is shown in the inset of the same figure.

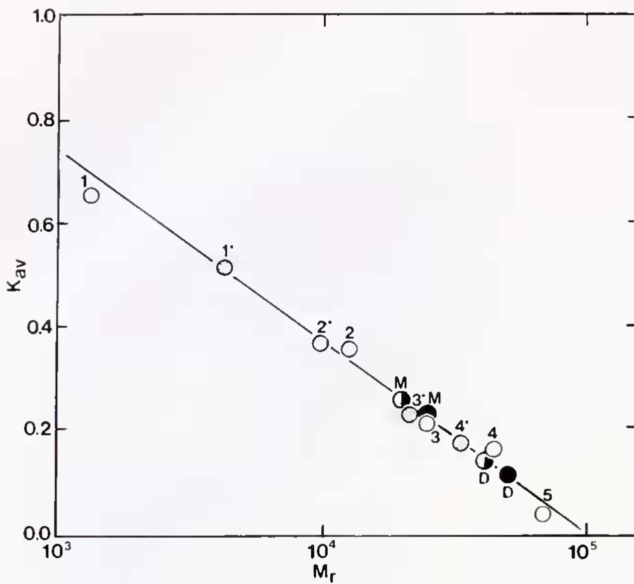

Figure 4. Calibration plot used to determine the molecular mass of the sperm proteins determined on a superose 12 HR 10/30 column under the elution conditions described in Figure 3A. Globular ○ and nonglobular ● proteins were used as standards. 1: Vitamin B 12 (Mr: 1,350 Da); 2: ribonuclease A (Mr: 13,200 Da); 3: chymotrypsingen A (Mr: 25,000 Da); 4: ovoalbumin (Mr: 46,000 Da); 5: bovine serum albumin (Mr: 68,000 Da); 1*: protamine salmine (Mr: 4,300 Da); 2*: protein PL-I from *Mytilus edulis* (Mr: 9,600 Da); 3*: histone H1 from calf thymus (Mr: 22,000 Da); 4*: Protein PL-I from *Spisula solidissima* (Mr: 33,000 Da). M = monomer form and D = dimer form of the major sperm protein components of: *Mytillineria nuttalli* ●, and *Agriodesma saxicola* ◑.

To increase the resolution in the separation, the 0.4 *N* HCl protein extracts were fractionated by reverse-phase HPLC; the elution profile is shown in Figure 2B. Although two peaks corresponding to each of the two major components could be clearly separated, both of them exhibited different amounts of what appeared to be overlapping cross-contamination.

Size fractionation of the starting HCl extracts by gel filtration under denaturing conditions in the presence of 6 *M* guanidinium chloride (Gdn-HCl) is shown in Figure 3. Although the peaks could not be completely resolved, the sample was partially fractionated as shown in Figure 3B. Indeed, when some of the eluting fractions from the different regions corresponding to the two major protein components were pooled together and rerun under the same conditions, two distinct peaks could then be clearly resolved. This was used as a basis for estimating the molecular mass of each of these protein components. Nevertheless, when the fraction under each separate peak was analyzed by urea acetic acid PAGE, the same cross-contamination observed in Figure 2B was again observed, although to a lesser extent (results not shown).

Characterization of the sperm-specific nuclear proteins from A. saxicola *and* M. nuttalli

The fractionation problems described in the preceding section began to be elucidated when the molecular mass of these proteins was analyzed. Figure 4 shows the calibration plot used to estimate the molecular mass of proteins from gel filtration analysis. The molecular mass of the two major protein components of the sperm nuclei in *A. saxicola* were: 21,000 Da for the fastest protein component and 43,000 Da for the slowest moving fraction. The values were 25,000 Da and 49,000 Da for *M. nuttalli*. These results suggested a monomer-dimer relationship between the slow and the fast moving protein fractions present in each species. To analyze this relationship, and to examine the nature of the association phenomenon, I incubated the crude HCl extracts in the presence of either β-mercaptoethanol or copper phenanthroline. Figure 5 shows the results of these treatments in the case of *A. saxicola*, and identical results were obtained with *M. nuttalli* (results not shown). The slower moving band completely disappears under reducing conditions for cysteine. Under oxidizing conditions (in the presence of copper phenanthroline) the relative intensity of the faster moving band (see Fig. 5b) slightly decreases, and higher association complexes are formed (see arrow in Fig. 5b). We are therefore dealing with the association of the faster moving protein components. Although the association seems to involve primarily the formation of dimers, the number of cysteines present in the monomer

Figure 5. Urea-acetic acid polyacrylamide gel electrophoresis of the nuclear sperm specific proteins from *Agriodesma saxicola* under A: reducing (6% β-mercaptoethanol) or B: oxidizing (copper-phenanthroline) conditions. SS = starting sample. m, d = monomer and dimer of major sperm protein component (PL-I). X, X_2 = monomer and dimer of the minor sperm protein component. The arrowhead indicates the presence of higher association oligomers obtained upon oxidative treatment.

cannot be clearly ascertained from the above experiments. Thus, although the strong tendency toward dimer formation would suggest the presence only of one cysteine per molecule, the lack of complete dimerization observed in Figure 5b, and the presence of association complexes higher than dimers, would strongly suggest the presence of more than one cysteine. The presence of two cysteine residues per molecule, which could easily form an internal disulfide bond, would explain the incomplete polymerization of the monomer, otherwise expected under the oxidizing conditions used here (Fig. 5b).

To determine the number of cysteines, as well as to establish the amino acid composition of the major nuclear protein component of the sperm of *A. saxicola* and *M. nuttalli*, protein fractions such as those shown in the insets of Figure 2A and B were pyridylethylated before amino acid analysis. A β-lactoglobulin sample was simultaneously treated and analyzed to check for the completion of the reaction. The amino acid analyses clearly show (see Table I) that the proteins of both *A. saxicola* and *M. nuttalli* contain two cysteine residues per molecule. Comparison with the amino acid analyses of other PL proteins, reveals the PL-I nature of the major nuclear protein component of the sperm of the two species analyzed. Like other PL-I proteins (Ausió, 1986; Ausió, 1988; Jutglar *et al.*, 1991), these have an internal trypsin resistant core (Fig. 6).

Besides the major protein components M and D, we have also characterized the minor component X of *A. saxicola*. This protein exhibits an amino acid composition that is almost identical to PL-I (see Table I). Although the amino acid analysis was carried out without prior pyridilethylation, trace amounts of cysteine could still be detected. Because of its relative electrophoretic mobility, X_2 (see Fig. 1-AS) most probably represents the dimer form of X. Indeed, X_2 disappears upon β-mercaptoethanol treatment of the starting protein sample (see Fig. 5).

Table I

Amino acid analysis (mol %) of the nuclear sperm-specific PL-I proteins of Agriodesma saxicola *PL-I (AS) and* Mytilimeria nuttalli *PL-I (MN) in comparison to those of* Spisula solidissima *PL-I (SS) (Ausió and Subirana, 1982a) and* Macoma nasuta *PL-I (MC) (Ausió, 1988)*

	PL-I (AS)	PL-I (MN)	PL-I (SS)	PL-I (MC)	X (AS)
Lys	18.7	16.3	24.8	21.8	15.1
His	—	0.4	—	2.3	0.4
Arg	34.8	33.8	23.1	26.9	34.7
Asx	1.5	1.8	0.6	0.8	2.5
Thr	2.0	1.0	4.3	4.0	1.6
Ser	20.8	26.5	21.7	20.2	21.4
Glx	1.1	0.8	0.6	0.8	4.6
Pro	0.5	0.8	2.4	1.8	1.7
Gly	6.0	5.5	3.0	2.2	5.0
Ala	3.3	4.2	14.2	11.3	4.4
1/2 Cys	0.9	0.6	—	0.7	tr.*
Val	3.2	2.4	2.3	2.4	2.8
Met	—	0.2	0.4	0.2	—
Ile	1.2	0.9	0.5	1.4	1.0
Leu	3.6	3.3	1.7	2.1	2.6
Tyr	1.5	1.0	0.3	0.5	1.0
Phe	0.7	0.7	0.3	0.7	1.0
Trp	—	—	0.3	—	

* Determination carried out in the absence of pyridilethylation treatment.

The amino acid analysis of the minor protein component X of *A. saxicola* (AS) is also shown.

Figure 6. Analysis of the time course of digestion by trypsin of the monomer (M) and dimer (D) of the PL-I protein of *Agriodesma saxicola*. Digestions were carried out in 2 *M* NaCl, 50 m*M* Na phosphate (pH 6.8), at an enzyme:substrate ratio of 1:500. The digestion times were: 0: 0 min; 1: 5 min; 2: 15 min; 3: 30 min; 4: 60 min and 5: 120 min. AS*: nuclear sperm-specific proteins of *A. saxicola* slightly degraded by an egg protease (see legend to Fig. 7). r: peptide resistant to digestion by egg proteases.

Specific degradation of PL-I in A. saxicola

Every specimen of *A. saxicola* analyzed was hermaphroditic. Although the male and female gonads are completely separated, some contamination of the sperm by oocytes sometimes occurred when accidental incisions were made in the ovary as the shells were being opened. The protein composition of the crude HCl nuclear extracts thus obtained showed a complex and highly variable pattern in urea acetic acid gel electrophoresis. Figure 7 shows a light microscope and electrophoretic analysis of several sperm samples with different amounts of contamination by oocytes. In preparations containing pure sperm, the electrophoretic analysis showed two major bands, M and D (Fig. 7a), corresponding to the monomer and dimer of PL-I, as well as a 15–20% of X and X_2. As the extent of contamination by female germinal cells increases (see Fig. 7b, c), the amounts of M and D present in the HCl extracts

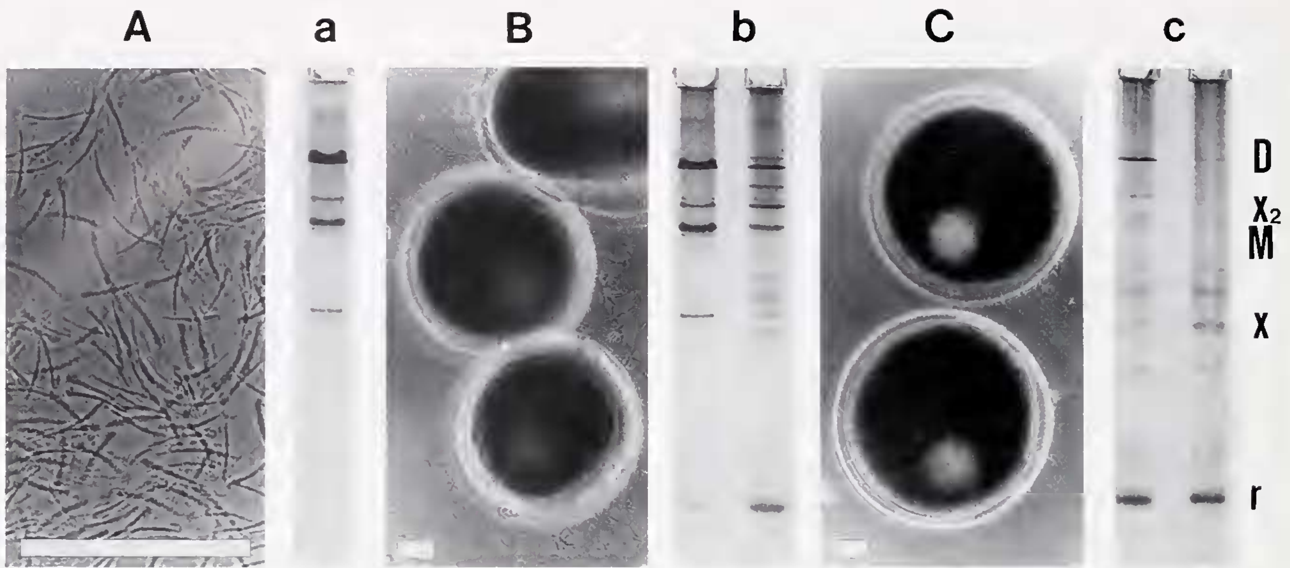

Figure 7. Microscopic analysis with phase contrast of pure sperm (A), and of sperm preparations containing an increasing amount of contamination by eggs (B) and (C). The samples were obtained from different specimens of *Agriodesma saxicola*. a, b, and c: electrophoretic analysis, in urea-acetic acid PAGE, of sperm preparations containing an increasing amount of contamination by eggs. As contamination increases, an extensive degradation of both the monomer (M) and dimer (D) forms of the major nuclear sperm-specific PL-I component is observed. A relatively resistant peptide—r—is generated during this degradation process. The white bar corresponds to 50 μm. X and X_2 are as in Figure 5.

decrease, and the proteins finally disappear completely. This is accompanied by the appearance of a complex pattern of new bands with faster electrophoretic mobility (Fig. 7b, c). Such protein pattern transition is clearly indicative of a degradation process elicited by specific proteases from the contaminating eggs. A similar *in vitro* degradation of sperm histones by the cytoplasm of sea urchin eggs has also been reported (Betzalel and Moav, 1987). A quite resistant degradation peptide, designated r, is produced during this process. Although the composition and nature of peptide r are completely unknown, it is certainly much smaller than the trypsin-resistant peptide obtained under *in vitro* conditions (see Fig. 6). A nuclear HCl protein extract from pure oocytes contained none of the proteins observed in Figure 7 (results not shown).

Discussion

In this work I have analyzed the protein composition of the nuclei of the sperm of two representatives of the subclass Anomalodesmata within the class Bivalvia. In both of the species analyzed—*Agriodesma saxicola* and *Mytilimeria nuttalli*—80–90% of the nuclear sperm-specific proteins consist of a mixture of dimer (D) and monomer (M) forms of a protamine-like (PL-I) protein. The remaining 10–20% includes the minor protein fractions X and Y.

The protamine-like nature of the major proteins is revealed by their amino acid composition (see Table I). They clearly fulfill the compositional definition of protamines (Subirana, 1983): (Lys + Arg) = 45–80%, (Ser + Thr) = 10–25%. Indeed, of all the PL proteins characterized so far, the ones analyzed here exhibit the highest arginine content within the PL classification (Ausió, 1986). The presence of a trypsin-resistant core (see Fig. 6) indicates that these proteins are also related to the proteins of the histone H1 family. Therefore, the PL major components of both *A. saxicola* and *M. nuttalli* should be undoubtedly classified within the PL-I class (Ausió, 1986). The presence of cysteine in these proteins does not seem to be an unusual feature; indeed, two cysteines also occur in the PL-I component of *Macoma nasuta* (Ausió, 1988).

Because the minor protein component X of *A. saxicola* has an amino acid composition indistinguishable from PL-I, these two proteins must be closely related, and X_2 (see Fig. 1-AS) may represent a dimer form of X. The same observations apply to the X and Y components of *M. nuttalli* (results not shown). The structural relationships among PL-I and the X and Y fractions remain obscure, but the similarity of their amino acid analyses to that of the major protein component suggests that X and Y may be proteolytic peptides from PL-I. They could arise from the activity of either a nuclear or an acrosomal sperm protease (Müller-Esterl and Fritz, 1981) during protein extraction. However, they do not seem to be related to any of the protein fragments produced by the protease digestion resulting from oocytic contamination, because

Table II

Classification of the class Bivalvia according to their protamine-like group (Ausió, 1986)

Subclass (a)	Representative species	Protein type (b)	Reference
Cryptodonta	—		—
Palaeotaxodonta			
Palaeoheterodonta	*Anodonta pisciniallis*	I (?)	(c)
Heterodonta	*Spisula solidissima*	I	(d)
	Ensis minor	II	(e)
	Macoma masuta	III	(f)
	Mytilus edulis	IV	(g)
Pteriomorphia	*Crassostrea gigas*	O	(h)
Anomalodesmata	*Agriodesma saxicola*	II	(i)
	Mytilimeria nuttalli	I	(i)

(a) According to Barnes (1980).
(b) According to Ausió (1986).
(c) Rozov *et al.* (1984).
(d) Ausió and Subirana (1982a).
(e) Giancotti *et al.* (1983).
(f) Ausió (1988).
(g) Ausió and Subirana (1982c).
(h) Sellos (1985).
(i) This work.

the presence of X_2 or X does not increase as the level of egg-induced degradation increases (see Fig. 7). Indeed, when whole sperm cells (without any prior preparation of the nuclei) were extracted with HCl for ½ h at 4°C immediately after sperm collection, the overall protein pattern observed was undistinguishable from the pattern of the HCl extracts prepared from nuclei uncontaminated by oocytes. In particular, the X-band was still observed.

The presence of a major PL-I protein in the sperm of the two species analyzed here would allow us to classify them within the protamine-like group I of my earlier classification of the bivalve mollusks (Ausió, 1986; see Table II). Species fulfilling this compositional pattern have also been described in other subclasses, including Palaeoheterodonta and Heterodonta. The presence of a PL-I protein, however, seems to be a common feature to all the species of the class Bivalvia (Jutglar *et al.*, 1991).

All of the PL-I proteins that have been analyzed in detail have structures related to the histone H1 superfamily (Ausió *et al.*, 1987; Ausió, 1988; Jutglar *et al.*, 1991). The structural similarities of PL-I to both histone H1 and the arginine-rich protamines from the vertebrates suggests a close evolutionary relationship between these proteins. In this sense, the increase in arginine and the decrease in lysine and alanine observed in the case of the PL-I proteins analyzed in the present work, when compared to other PL-I proteins, would indicate a further departure from their H1 nature and a closer relationship to the protamines from vertebrates.

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Literature Cited

Ando, T., M. Yamasaki, and K. Suzuki. 1973. P. 28 in *Protamines.* Vol. 12 of *Molecular Biology, Biochemistry and Biophysics.* Springer-Verlag, Berlin, Heidelberg, NY.

Ausió, J., and J. A. Subirana. 1982a. A high molecular weight nuclear basic protein from the bivalve molluscs. *J. Biol. Chem.* **257:** 2802–2805.

Ausió, J., and J. A. Subirana. 1982b. Conformational study and determination of the molecular weight of highly charged basic proteins by sedimentation equilibrium and gel electrophoresis. *Biochemistry* **21:** 5910–5918.

Ausió, J., and J. A. Subirana. 1982c. Nuclear proteins and the organization of chromatin in spermatozoa of *Mytilus edulis. Exp. Cell. Res.* **141:** 39–45.

Ausió, J. 1986. Structural variability and compositional homology of the protamine-like components of the sperm from the bivalve molluscs. *Comp. Biochem. Physiol.* **85B:** 439–449.

Ausió, J., A. Toumadje, R. McParland, R. R. Becker, W. C. Johnson, Jr., and K. E. van Holde. 1987. Structural characterization of the trypsin resistant core in the nuclear sperm-specific protein from *Spisula solidissima. Biochemistry* **26:** 975–982.

Ausió, J. 1988. An unusual cysteine-containing histone H1-like protein and two protamine-like proteins are the major nuclear proteins of the sperm of the bivalve mollusc: *Macoma nasuta. J. Biol. Chem.* **263:** 10,141–10,150.

Barnes, R. D. 1980. *Invertebrate Zoology,* 4th ed. Saunders College, Philadelphia.

Bernard, F. R. 1983. Catalogue of the living Bivalvia of the eastern Pacific Ocean: Bering Strait to Cape Horn. *Canadian Special Publication of Fisheries and Aquatic Sciences* **81:** 83.

Betzalel, M., and B. Moav. 1987. Degradation of sperm histones *in vitro* by cytoplasm of the sea urchin egg. *Cell Differ.* **20:** 125–136.

DeLange. 1976. *Handbook of Biochemistry and Molecular Biology.* 3rd ed. (Proteins), G. Fasman, ed. CRC press Boca Raton, FL, vol II, 294 pp.

Giancotti, V., E. Russo, M. Gasparini, D. Serrano, D. Del Piero, A. W. Thorne, P. D. Cary, and C. Crane-Robinson. 1983. Proteins from the sperm of the bivalve mollusc: *Ensis minor. Eur. J. Biochem.* **136:** 509–516.

Hirs, C. M. W. 1967. Determination of cysteine as cysteic acid. *Methods Enzymol.* **XI:** 59–62.

Jutglar, L., J. I. Borell, and J. Ausió. 1991. Primary, secondary and tertiary structure of the core of a histone H1-like protein from the sperm of *Mytilus. J. Biol. Chem.* **266:** 8184–8191.

Kasinsky, H. E. 1989. Specifity and distribution of Sperm Basic Proteins. Pp. 73–163 in *Histones and Other Basic Nuclear Proteins,* Vol. I, L. S. Hnilica, G. S. Stein, and J. L. Stein, eds. CRC Press, Boca Raton, FL.

Kuehl, J. 1979. Synthesis of high mobility group proteins in regenerating rat liver. *J. Biol. Chem.* **254:** 7276–7281.

Mogensen, C., S. Carlos, and J. Ausió. 1991. Microheterogeneity and interspecific variability of the nuclear sperm proteins from *Mytilus*. *FEBS Lett.* **282:** 273–276.

Müller-Esterl, W., and H. Fritz. 1981. Sperm acrosin. *Methods Enzymol.* **80:** 621–632.

Odintsova, N. A., S. M. Rozov, and I. A. Zalenskaya. 1989. The chromosomal proteins from the sperm of the bivalve molluscs *Swiftopecten swifty* and *Glycymerys yesonensis*. *Comp. Biochem. Physiol.* **93B:** 163–167.

Rozov, S. M., V. A. Brednikov, F. K. Gorel, M. V. Lavrenteva, and L. P. Solonenko. 1984. Structure of lysine-rich histone from sperm of *Anodonta piscinalis*. *Mol. Biol.* (transl) (USSR) **18:** 1497–1508.

Sellos, D. 1985. The histones isolated from the sperm of the oyster *Crassostrea gigas*. *Cell Differ.* **17:** 183–192.

Subirana, J. A., C. Cozcolluela, J. Palau, and M. Unzeta. 1973. Protamines and other basic proteins from spermatozoa of molluscs. *Biochim. Biophys. Acta* **317:** 364–379.

Subirana, J. A. 1983. *Nuclear Proteins in Spermatozoa and Their Interactions with DNA*, Pp. 197–214 in *The Sperm Cell*, J. André, ed. Martinus Nijhoff B. V., The Hague.

Subirana, J. A., and J. Colom. 1987. Comparison of protamines from freshwater and marine bivalve molluscs: evolutionary implications. *FEBS Lett.* **220:** 193–196.

Zalensky, A. O., and I. A. Zalenskaya. 1980. Basic chromosomal proteins of marine invertebrates II. The proteins from the Bivalvia molluscs. *Comp. Biochem. Physiol.* **66B:** 415–419.